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Endocrine-Disrupting Chemicals (EDCs): *In Vitro* Mechanism of Estrogenic Activation and Differential Effects on ER Target Genes

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Abbreviations:

Estradiol (E2) -

Endocrine-Disrupting Chemicals (EDCs) -

Estrogen receptor α (ERα) -

Estrogen receptor β (ERβ) -

Estrogen responsive element (ERE) -

Abstract

Background: Endocrine-disrupting chemicals (EDCs) are shown to influence the activity of estrogen receptors (ERs) and alter the function of the endocrine system. However, the diversity of EDC effects and mechanisms of action are poorly understood.

Objectives: We identified agonistic activity of EDCs through ERα and ERβ and their effects on ER-mediated target genes.

Methods: HepG2 and HeLa cells were utilized to determine the agonistic activity of EDCs on ERα and ERβ via luciferase reporter assay. Ishikawa cells stably expressing ERα were used to determine changes in endogenous ER target gene expression by EDCs.

Results: Twelve EDCs were categorized into three groups based on their product class and similarity of chemical structure. Luciferase reporter analysis demonstrated that their ER agonistic effects are in a cell type/promoter specific manner. Bisphenol A, Bisphenol AF and 2-2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (Group 1) strongly activated the ERα ERE-mediated responses. Daidzein, Genistein, Kaempferol and Coumestrol (Group 2) activated both the ERα and ERβ ERE-mediated activities. Endosulfan and Kepone (Group 3) weakly activated ERα. Only a few EDCs significantly activated the "tethered" mechanism via ERα or ERβ. Real time-PCR results indicated that Bisphenol A and Bisphenol AF consistently activated endogenous ER target genes, but the activities of other EDCs on ER target gene expression changes were compound specific.

Conclusion: EDCs with similar chemical structures tended to have comparable ERα and ERβ ERE-mediated activities, but did not correlate with their previously reported ligand binding affinities. Using ERα stable cells, we show EDCs differentially induce endogenous ER target gene activities.

Introduction

Many natural and synthetic chemicals are reported to disrupt the normal function of the endocrine system (Henley and Korach 2010). These compounds, classified as endocrine-disrupting chemicals (EDCs), interfere with hormone biosynthesis, metabolism or action, which can result in deviation from normal homeostatic control and can alter normal development and reproduction (Diamanti-Kandarakis et al. 2009). Many known EDCs influence the activity of the estrogen receptors (ERs) and alter their function in *in vitro* and *in vivo* model systems (Diamanti-Kandarakis et al. 2009). Estrogens play an essential role in the growth, differentiation, and homeostasis of a number of target tissues, including the male and female reproductive tracts, mammary glands, bone, brain and liver (Katzenellenbogen 1996; Katzenellenbogen et al. 1997; Lubahn et al. 1993; McDonnell and Norris 2002; Nilsson et al. 2001; Pettersson and Gustafsson 2001). The biological effects of estrogen (E2) are mediated through two ERs, ERα and ERβ, which belong to the nuclear receptor super-family of ligand-inducible transcription factors (Hall and McDonnell 2005). There are two major mechanisms of ER-mediated transcriptional gene regulations. In the classical mechanism, ERs directly bind to estrogen response elements (EREs) located in the promoter region of target genes. The non-classical mechanism is the “tethered” mechanism, which involves the ERs regulating gene expression by associating with other transcription factors such as c-Jun and c-Fos which bind the DNA, but without direct ER-DNA binding (Bjornstrom and Sjoberg 2005; Hall and McDonnell 2005; O'Lone et al. 2004).

Estrogens regulate a large number of target genes through the ER. *PR* (progesterone receptor) and *pS2* are the well-known ER target genes (Berry et al. 1989; Katzenellenbogen 2000). *GREB1* (gene regulation by estrogen in breast cancer 1) and *SPUVE* (a member of the trypsin family of serine proteases) have been reported as ER-responsive genes (Henley et al. 2009; Reid et al.

2005). Recently, we discovered that these target genes are induced by Bisphenol A (BPA) and Bisphenol AF(BPAF), a fluorinated derivative of BPA, and the gene expression changes are compound specific (Li et al. 2012). *WISP2* (WNT1-inducible-signaling pathway protein 2) gene expression is enhanced by important modulators of human breast cancer cell proliferation such as E2, progesterone, and epidermal growth factor. These effects, inhibited by appropriate antagonists, indicate that steroids and growth factor-induced upregulation of *WISP-2* may be mediated through ERs (Dhar et al. 2007). *SDF-1* (stromal cell-derived factor 1) was identified as a key target of estrogens in ER-positive breast and ovarian cells (Hall and Korach 2012). The correlation between chemical structure and functionality of the EDCs through the ERs and their effects on ER target genes remains unclear.

BPA, BPAF and EDCs with a similar chemical structure have been frequently studied. BPA is widely used in the manufacturing of polycarbonate plastics and as a non-polymer additive to other plastics (Wetherill et al. 2007). BPA uptake in human from food, beverages, and the environment are measured in adult and fetal serum with a range of 0.5-40 nM (Welshons et al. 2006). BPAF is used in polycarbonate copolymers in high-temperature composites, electronic materials, and specialty polymer applications (Akahori et al. 2008; Perez et al. 1998). 2,2-bis(p-Hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is an estrogenic metabolite of the pesticide methoxychlor and has similar estrogenic effects as BPA (Borgeest et al. 2002; Hewitt and Korach 2011; Klotz et al. 2000). 4-n-Nonylphenol (4n-NP) is a resistant alkylphenol that is degraded from alkylpheol ethoxylates and is generally present in food (Guenther et al. 2002; Ying et al. 2002).

A number of natural products (known as phytoestrogens) are identified as estrogenic EDCs. Daidzein (Dai) is a soy-derived isoflavone that originates from plants and herbs (Dang 2009).

Genistein (Gen) is also an isoflavone found in a number of plants including lupin, fava beans, soybeans, kudzu and psoralea (Dang 2009). Kaempferol (Kaem) is a flavonoid/isoflavone isolated from tea, broccoli, grapefruit, apples and other plant sources (Calderon-Montano et al. 2011). Apigenin (Api) is a flavonoid/flavone used to dye wool (Ferreira et al. 2006). Coumestrol (Coum) is an organic compound in the class of phytochemicals known as coumestans and has classically been categorized as a phytoestrogen due to its binding to the ER (Markaverich et al. 1995).

Other estrogenic EDCs of interest with a common structural component include Endosulfan (Endo), Kepone (Kep), and 1-Bromopropane (1-BP). Endo is a fluorinated organic insecticide and the animal studies indicate that it affects the male reproductive system (Murray et al. 2001). Kep, also known as chlordecone, is a chlorinated polycyclic hydrocarbon insecticide and fungicide. *In vitro* studies show that Kep had ligand binding affinity to ERα (van Lipzig et al. 2004). 1-BP is categorized as a high-production-volume chemical and is used in the manufacture of pharmaceuticals, pesticides, and other chemicals (Anderson et al. 2010).

In this study, we used two ER negative cell lines, HepG2 and HeLa to analyze the effects of twelve estrogenic EDCs, which were grouped based on chemical structure and product class, on the estrogenic ERE- and AP-1/Sp1-mediated responses of ERα and ERβ. Using Ishikawa cells stably expressing ERα, we evaluated changes in endogenous ER target gene expression after EDC treatment.

Materials and Methods

Chemicals. 17β-Estradiol (E_2) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and ICI 182,780 was from Tocris Bioscience (Ellisville, MO, USA). The twelve EDCs used in this study were provided by the Midwest Research Institute (Kansas City, MO, USA) via a contract with the National Toxicology Program (NTP). The chemical names, Chemical Abstracts Services Registry Number (CASRN) and the source are summarized in the Supplemental Material, Table s1.

Plasmids. pcDNA vector plasmid was purchased from Promega (Madison, WI, USA), pRL-TK vector plasmid from Invitrogen (Carlsbad, CA, USA) and 7xAP-1 Luc from Stratagene (La Jolla, CA, USA). pcDNA/mouse WT-ERα (pcDNA/ERα) and pcDNA/ΔNmERβ310G (former pcDNA/mouse WT-ERβ) have been described previously (Mueller et al. 2003). Full-length mouse ERβ expression plasmid, pcDNA/WT-ERβ, was generated as described in the Supplemental Material. The luciferase reporters 3xERE and pS2ERE have been described previously (Hall et al. 2002). The following reporters were gifts: pRSV/c-Jun (M. Karin, UCSD), -73Col AP-1 Luc (D. McDonnell, Duke U) and p21Sp1 Luc (L. Jameson, U of Penn).

Cell lines and tissue culture. The human hepatocellular cancer cell line HepG2 and cervical epithelial cancer cell line HeLa (both ER negative) were purchased from ATCC (Manassas, VA, USA). Human endometrial adenocarcinoma stable cell lines Ishikawa/vector (Ishikawa/vec) and Ishikawa/WT ERα (Ishikawa/ERα) have been described previously (Burns et al. 2011; Li et al. 2012). HepG2 cells were maintained in phenol red free Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gemini Bio Products, West Sacramento, CA, USA) and 4 mM L-glutamine (Invitrogen, Carlsbad, CA,

USA). HeLa cells were maintained in phenol red free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 4 mM L-glutamine. The stable cell lines Ishikawa/vec and Ishikawa/ERα were maintained in phenol-red free DMEM:F12 medium (DMEM:F12, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and Geneticin (G418, 1 mg/mL, Invitrogen, Carlsbad, CA, USA). For serum starved conditions, 10% HyClone Charcoal/Dextran stripped FBS (sFBS, Thermo Scientific, Waltham, MA, USA) was substituted for FBS in the medium (starve medium).

Transient transfection and luciferase assay. Cells were seeded in 24-well plates with serum-starved medium overnight. A total of 0.5 μg of DNA, including 0.2 μg of expression plasmid, 0.2 μg of reporter plasmid and 0.1 μg of pRL-TK plasmid, were transfected overnight by using the Effectene transfection reagent (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. After changing to fresh starve medium for 8 hours, cells were treated with EDCs as described in the figure legends. For experiments with pRSV/c-Jun on 7xAP-1 Luc, cells were transfected with a total of 0.7 μg of DNA, including 0.2 μg of ERα or ERβ, 0.2 μg of pRSV/c-Jun, 0.2 μg of 7xAP-1 Luc and 0.1 μg of pRL-TK plasmids. Luciferase assays were performed using the Dual Luciferase Reporter Activity System (Promega, Madison, WI, USA). Transfection efficiency was normalized by renilla luciferase using pRL-TK plasmid. Fold changes were calculated relative to the vehicle. All experiments were repeated at least three times and data shown is representative of triplicates as fold increase calculated relative to the vehicle (control) ± standard error of the mean (SEM).

RNA extraction and real-time PCR. Cells were cultured in starve medium for 2 days and then treated with 10 nM E2 or 100 nM EDCs for 18 hours. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was performed

using Superscript reverse transcriptase according to the manufacturer's protocol. The mRNA levels of ER target genes were measured using SYBR green assays (Applied Biosystems, Carlsbad, CA, USA). The sequences of primers used in real-time PCR were as follows: for human *PR* (NM_000926.4): the forward primer 5'-GACGTGGAGGGCGCATAT-3', reverse primer 5'-GCAGTCCGCTGTCCTTTTCT-3'; for human *pS2/TFF1* (NM_003225.2): the forward primer 5'-GCCCTCCCAGTCTGCAAATA -3', reverse primer 5'-CTGGAGGGACGTCGATGGTA -3'; for human *GREB1* (NM_014668): the forward primer 5'-CAAAGAATAACCTGTTGGCCC-3', reverse primer 5'-GACATGCCTGCGCTCTCATAC-3'; for human *SPUVE* (NM_007173): the forward primer 5'-ATGCCCGAGCAGATGAAATT-3', reverse primer 5'-CCAACCCTTGGGCACATG-3'; for human *WISP2* (NM_003881): the forward primer 5'-TGAGCGGCACACCGAAGAC-3', reverse primer 5'ACAGCCATCCAGCACCAG-3'; for human *SDF-1* (NM_000609): the forward primer 5'-GTGGTCGTGCTGGTCCTC-3', reverse primer 5'-GATGCTTGACGTTGGCTCTG-3'. Cycle threshold (Ct) values were obtained using the ABI PRISM 7900 Sequence Detection System and analysis software (Applied Biosystems, Foster City, CA, USA). Each sample was quantified against its β-actin transcript content: the forward primer 5'-GACAGGATGCAGAAGGAGATCAC-3', reverse primer 5'-GCTTCATACTCCAGCAGG-3'. The experiments were repeated three times and results are presented as fold increase calculated relative to the vehicle (control) of Ishikawa/vec cells ± SEM.

Statistical Analysis. One-way ANOVA with Dunnett's Multiple Comparison test and Two-way ANOVA with Bonferroni post-tests were performed using GraphPad Prism version 6.00 (San Diego, CA, USA).

Results

EDCs categorize into three groups by similarities of chemical structure. Twelve EDCs were categorized into three groups based on the chemical and product classes (Figure 1 and Table 1). Group 1 consists of BPA, BPAF, HPTE and 4n-NP due to their shared bisphenol or phenol group. Group 1 EDCs are widely used as chemical intermediates. A group of EDCs from natural products, including Dai, Gen, Kaem, Api and Coum comprise Group 2 since they each contain flavonoid, isoflavone or phenol. Additionally, Endo, Kep and 1-BP belong to Group 3 because they each contain organochlorine or organobromine in their chemical structures. Group 3 EDCs have traditionally been used as pesticides or chemical intermediates.

ERE-mediated estrogenic activation of ER□ and ER□ by EDCs. To evaluate the ERE-mediated transcriptional activity of ERα and ERβ, we examined promoter activation in two ER negative cell lines, HepG2 and HeLa. The two luciferase reporters, 3xERE (modified reporter) and pS2ERE (endogenous pS2 gene reporter) (Hall et al. 2002) were used to determine the differential effects of these EDCs. First, we confirmed that there was no reporter activation without ER expression in both cell lines when stimulated with 10 nM E2 (data not shown). Because estrogenic effects of BPA and BPAF were seen at 100 nM with WT-ERα (Li et al. 2012), all EDCs were examined at this concentration.

The ERα ERE-mediated activation by EDCs is shown in Figure 2A. HepG2 cells were highly responsive to E2, with up to 50-fold increases in 3xERE-mediated transactivation (Figure 2A, top left). Group 1 and Group 2 EDCs strongly activated ERα 3xERE-mediated responses in HepG2 cells, with the exception of 4n-NP and Api at 100 nM. However, no activation was seen with Group 3 EDCs at 100 nM concentration. Even though the pS2ERE reporter had weaker

response to E2, similar responses were obtained with EDC treatments (Figure 2A, top right). Interestingly, induction with Endo in HepG2 cells was detected only with the pS2ERE reporter. In HeLa cells, all EDCs, with the exception of Api and 1-BP, significantly induced 3xERE-mediated activity (Figure 2A, bottom left). However, only three EDCs from Group 1 (BPA, BPAF and HPTE) and four EDCs from Group 2 (Dai, Gen Kaem and Coum) induced pS2ERE-mediated activation (Figure 2A, bottom right).

For ERβ ERE-mediated activation, both ERE reporters exhibited responses to E2 in HepG2 cells (Figure 2B, top). BPAF from Group 1 and Dai, Gen, Kaem and Coum from Group 3 have strong activation of ERβ 3xERE and pS2ERE-mediated responses in HepG2 cells. In HeLa cells, ICI, BPA and Api induced activity with the 3xERE reporter, and Dai and Coum induced activity with the pS2ERE reporter (Figure 2B, bottom). However, Group 3 EDCs did not activate ERβ ERE-mediated activity in HepG2 or HeLa cells. To confirm that the reporter activation of EDCs through ERα and ERβ was ER specific, ICI 182,780 (ICI), a pure ER antagonist, was used to block activity (data not shown). These results demonstrate that EDCs can activate ERE-mediated transcription in different cell types via ERα and ERβ in cell type and promoter selective manners, and that the structural similarities among the EDCs correlate to their estrogenic activity.

The effects of EDCs on AP-1 and Sp1 reporters for ERα and ERβ. To verify the effects of the EDCs on the "tethered" mechanism of ERα and ERβ, the 7xAP-1 reporter (Jakacka et al. 2001; Kushner et al. 2000; Webb et al. 1995), the -73Col AP-1 reporter (Sharma and Richards 2000) and the p21Sp1 reporter (De Siervi et al. 2004) were used to test the AP-1/Sp1-mediated activation.

In order to detect the ligand-dependent/AP-1-mediated reporter activity, the 7xAP-1 Luc reporter, c-Jun, and ERα or ERβ were co-transfected into the cells. For ERα activation, ICI, as a positive control (Kushner et al. 2000), had stronger response in HepG2 than in HeLa cells (Figure 3A). ICI induced the 7xAP-1 reporter activity over 10 fold in HepG2 cells; however, only BPA and 4n-NP showed weak activities (Figure 3A, top left). In HeLa cells, Kaem, Api and Coum (from Group 2) and all Group 3 EDCs activated the 7xAP-1 reporter (Figure 3A, bottom left). For ERβ, only ICI induced the 7xAP-1 reporter activity in HepG2 cells (Figure 3A, top right). All EDCs induced minor ERβ/7xAP-1 reporter activity in HeLa cells, but only Dai showed significant activation (Figure 3A, bottom right).

Using the -73Col AP-1 reporter in HeLa cells, only BPA (from Group 1) showed weak activity via ERα (Figure 3B, left) and ICI induced weak activity via ERβ (Figure 3B, right). However, there was no activation for either ER with the EDCs in HepG2 cells (data not shown). Lastly, for the p21Sp1 reporter using either ERα or ERβ, the induction levels were insignificant to discriminate agonistic tendencies in both HepG2 and HeLa cells (data not shown). These findings suggest that EDCs induce weak activity for the "tethered" mechanism in a cell type and promoter specific manner.

The effects of EDCs on expression of ER target genes. To characterize the ER dependent response of EDCs, we examined their effects on ERα target genes (*PR*, *pS2*, *GREB1*, *SPUVE*, *WISP2*, and *SDF-1*) using real time-PCR in Ishikawa/ERα stable cells (Burns et al. 2011; Li et al. 2012). Fold changes in gene expression, relative to vehicle as a control, are shown in Figure 4. For Group 1 EDCs, BPA, BPAF and HPTE significantly induced the endogenous ERα target genes, *PR*, *pS2*, *GREB1*, *SPUVE*, *WISP2*, and *SDF-1*, with the exception of HPTE for *SDF-1*. 4n-NP only induced *WISP2* gene significantly. However, Group 2 EDCs varied in their induction

of ER target genes. Dai induced the *PR*, *pS2*, *GREB1*, *SPUVE*, and *SDF-1* genes, Gen induced the *PR*, *pS2*, *SPUVE*, and *WISP2* genes, Kaem induced the *PR*, *pS2* and *WISP2* genes, Api induced the *WISP2* and *SDF-1* genes, and Coum induced the *PR*, *WISP2*, and *SDF-1* genes significantly. Similarly, Group 3 EDCs varied in their induction of target genes; Endo activated *pS2*, *GREB1* and *WISP2*, Kep only activated *WISP2*, and 1-BP activated *WISP2* and *SDF-1* significantly. In contrast, expression of target genes in the Ishikawa/vector stable cells did not change with any EDC treatments, demonstrating that the changes in target gene expression are ER dependent. These results indicate that EDCs have effects on many aspects of transcriptional regulation in this *in vitro* cell culture model, and this information may be helpful in identifying compound specific genes that are involved in cellular signaling responses.

Discussion

EDCs activate the classical ER molecular mechanism (ligand- and ERE-mediated ER activation) in a manner correlative to chemical structure similarity. Many EDCs adversely impact estrogen signaling by interacting with two ERs: ERα and ERβ. We are interested in defining the roles of the ERs in mediating cellular and physiological responses to EDCs based on similarities in chemical structure. One of the most significant findings of this study is that the structural similarities of the EDCs correlate with their estrogenic activity for ERs. The 3xERE Luc reporter contains a series of three 13 base pair inverted repeats, GGTCAnnnTGACC (perfect ERE), while the pS2ERE Luc, derived from the human pS2 gene promoter, contains an imperfect ERE sequence, GGTCAnnnTGGCC, and several AP-1 sites (Hall et al. 2002). Using these two reporters, we found that BPA, BPAF and HPTE (Group 1) strongly activated ERα ERE-mediated responses, but the same compounds did not activate ERβ. Evidence shows that BPA binds

strongly to estrogen-related receptor γ (ERR-γ), an orphan receptor that behaves as a constitutive activator of transcription, but only weakly binds to the ERs (Matsushima et al. 2007). In contrast to our reporter assays, in vitro receptor-binding analysis shows that the ligand binding activity of BPAF and HPTE is three times stronger for ERβ than for ERα (Matsushima et al. 2010). With Group 2 EDCs, Dai, Gen, Kaem, and Coum activated both ERα and ERβ ERE-mediated activity. In fact, Dai, Gen, Kaem and Coum were more competitive than E2 for binding to ERβ (Hwang et al. 2006; Kuiper et al. 1998). These results indicate that the ERE-mediated activity of these EDCs does not correlate with their receptor ligand binding activity from in vitro data. Recent analysis indicated that formerly used mouse ERβ expression plasmid, in our study, had a mutation of 310 glutamic acid (E) to glycine (G) . Using this mutated ERβ plasmid, we found that BPAF (Group 1 EDC) and Kaem (Group 2 EDC) lost the majority of ERE-mediated activity in HepG2 cells relative to full-length ERβ (Supplemental material, Figure S1). Additionally, Endo and Kep (Group 3) exhibited weak activation of ERα in a cell type specific manner (only in HeLa cells), suggesting that cell type specific factors are involved in regulating ER ERE-mediated activity.

EDCs activate the non-classical “tethered” ER mechanism (AP-1/Sp1-mediated ER activation) in a manner not correlative to chemical structure similarity There is growing literature supporting E2’s ability to affect gene expression through the non-classical “tethered” mechanism, which involves ER modulating the activity of other transcription factors such as activator protein 1 (AP-1) and specificity protein 1 (Sp1). Webb et al. first reported the ER activation of the -73Col AP-1 promoter reporter construct, derived from the human collagenase promoter (Webb et al. 1995). Using three different reporters (7xAP-1, -73Col AP-1, p21Sp1 Luc), we found that ERα AP-1-mediated activation in HeLa cells was variable; Kaem, Api and Coum (from Group 2) and

Endo, Kep and 1-BP (from Group 3) had activity with the 7xAP-1 reporter. In contrast, all EDCs induced minor activity for the ERβ "tethered"-mediated mechanism with the 7xAP-1 reporter in HeLa cells, but only Dai showed significant activation. Furthermore, there was no activation with any EDCs with the -73Col AP-1 reporter via ERα or ERβ (with the exception of BPA in HeLa and ICI in HepG2 cells). Our data suggest that cell specific co-regulators may be involved in reporter activation by the EDCs in these different cell types. Additionally, ER AP-1-mediated activation was not seen in HepG2 cells with most of the EDCs (with the exception of ICI). Similar results were obtained with the mutated ERβ (Supplemental material, Figure s2). These data indicated that EDCs activate the non-classical "tethered" ER mechanism in a manner not correlative to their chemical structure similarity and that ERβ AP-1-mediated activation of EDCs occurs only in a cell type/promoter specific manner.

EDCs induce ER target gene expression in a compound specific manner. ERs, as transcription factors, are able to induce gene expression events sufficient for altered cellular responses, some of which include cell division and cancer progression. The advent of expression microarrays allowed for the investigation of global gene expression changes after ligand treatment. Our laboratory has reported gene expression profiles of the estrogenic activity of BPA and HPTE in the mouse uterus. The results showed that similar target genes are induced by BPA, HPTE, and E2 two hours after treatment (Hewitt and Korach 2011). This demonstrates that there may be similar target genes in the uterus that are activated by EDCs and E2. Because the sequences of the DNA binding domains (DBDs) of ERα and ERβ are 97% similar and ligand binding induces conformational changes to the ERs, promoting dimerization and high-affinity binding to EREs within the regulatory regions of target genes (Hall and McDonnell 2005), we used the Ishikawa cells stably expressing ERα (Burns et al. 2011; Li et al. 2012) to investigate several endogenous

ER target genes, including PR, pS2, GREB1, SPUVE, WISP2, and SDF-1 after EDC treatments. We found that E2 induced expression of PR, pS2 and GREB1genes in this in vitro model. Our results showed that BPA, BPAF and HPTE (Group 1) induced all six of the endogenous genes significantly, with the exception of SDF-1 by HPTE treatment. However, induction of target gene expression by Group 2 and Group 3 EDCs was target gene specific. More interestedly, ICI induced the two ER target genes, WISP2 and SDF-1, suggesting that WISP2- or SDF-1 genes may have AP-1 type regulating sequence. Future analysis of specific target gene promoters would be beneficial in understanding any similarities or differences of how the EDC's activate the ERs and elicit tissue specific actions.

Conclusions

In this study, one of the most significant findings is that there appears to be a correlation between EDCs with similar chemical structure and their ERE-mediated activities for both ERα and ERβ, but not their known ligand binding affinities. Only a few EDCs tested in this study weakly induce ERα and ERβ via the "tethered"-mediated mechanism. Using cells stably expressing ERα, we demonstrated that multiple EDCs can differentially induce endogenous ER target genes. Taken together, these data raise a question as to whether multiple assays will be required to assess the potential activity of EDCs. Our results also demonstrate the mechanistic importance of chemical structure similarities and cell type/promoter specificity in the evaluation of potential activities of multiple EDCs.

References

Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. 2008. Relationship between the results of in vitro receptor binding assay to human estrogen receptor alpha and in vivo uterotrophic assay: comparative study with 65 selected chemicals. Toxicol In Vitro 22(1):225-231.

Anderson SE, Munson AE, Butterworth LF, Germolec D, Morgan DL, Roycroft JA, et al. 2010. Whole-body inhalation exposure to 1-bromopropane suppresses the IgM response to sheep red blood cells in female B6C3F1 mice and Fisher 344/N rats. Inhalation Toxicol 22(2):125-132.

Berry M, Nunez AM, Chambon P. 1989. Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci U S A 86(4):1218-1222.

Bjornstrom L, Sjoberg M. 2005. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19(4):833-842.

Borgeest C, Greenfeld C, Tomic D, Flaws JA. 2002. The effects of endocrine disrupting chemicals on the ovary. Front Biosci 7:d1941-1948.

Burns KA, Li Y, Arao Y, Petrovich RM, Korach KS. 2011. Selective mutations in estrogen receptor alpha D-domain alters nuclear translocation and non-estrogen response element gene regulatory mechanisms. J Biol Chem 286(14):12640-12649.

Calderon-Montano JM, Burgos-Moron E, Perez-Guerrero C, Lopez-Lazaro M. 2011. A review on the dietary flavonoid kaempferol. Mini Rev Med Chem 11(4):298-344.

Dang ZC. 2009. Dose-dependent effects of soy phyto-oestrogen genistein on adipocytes: mechanisms of action. Obes Rev 10(3):342-349.

De Siervi A, Marinissen M, Diggs J, Wang XF, Pages G, Senderowicz A. 2004. Transcriptional activation of p21(waf1/cip1) by alkylphospholipids: role of the mitogen-activated protein kinase pathway in the transactivation of the human p21(waf1/cip1) promoter by Sp1. Cancer Res 64(2):743-750.

Dhar K, Banerjee S, Dhar G, Sengupta K, Banerjee SK. 2007. Insulin-like growth factor-1 (IGF-1) induces WISP-2/CCN5 via multiple molecular cross-talks and is essential for mitogenic switch by IGF-1 axis in estrogen receptor-positive breast tumor cells. Cancer Res 67(4):1520-1526.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 30(4):293-342.

Ferreira CV, Justo GZ, Souza AC, Queiroz KC, Zambuzzi WF, Aoyama H, et al. 2006. Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives. Biochimie 88(12):1859-1873.

Guenther K, Heinke V, Thiele B, Kleist E, Prast H, Raecker T. 2002. Endocrine disrupting nonylphenols are ubiquitous in food. Environ Sci Technol 36(8):1676-1680.

Hall JM, Korach KS. 2012. Endocrine disrupting chemicals promote the growth of ovarian cancer cells via the ER-CXCL12-CXCR4 signaling axis. Mol Carcinog Apr 30. doi:10.1002/mc.21913.

Hall JM, McDonnell DP. 2005. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. Mol Interventions 5(6):343-357.

Hall JM, McDonnell DP, Korach KS. 2002. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. Mol Endocrinol 16(3):469-486.

Henley DV, Korach KS. 2010. Physiological effects and mechanisms of action of endocrine disrupting chemicals that alter estrogen signaling. Hormones (Athens) 9(3):191-205.

Henley DV, Mueller S, Korach KS. 2009. The short-chain fatty acid methoxyacetic acid disrupts endogenous estrogen receptor-alpha-mediated signaling. Environ Health Perspect 117(11):1702-1706.

Hewitt SC, Korach KS. 2011. Estrogenic activity of bisphenol A and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) demonstrated in mouse uterine gene profiles. Environ Health Perspect 119(1):63-70.

Hwang CS, Kwak HS, Lim HJ, Lee SH, Kang YS, Choe TB, et al. 2006. Isoflavone metabolites and their in vitro dual functions: they can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J Steroid Biochem Mol Biol 101(4-5): 246-253.

Jakacka M, Ito M, Weiss J, Chien PY, Gehm BD, Jameson JL. 2001. Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. J Biol Chem 276(17):13615-13621.

Katzenellenbogen BS. 1996. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol Reprod 54(2):287-293.

Katzenellenbogen BS. 2000. Mechanisms of action and cross-talk between estrogen receptor and progesterone receptor pathways. J Soc Gynecol Investig 7(1 Suppl):S33-37.

Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM. 1997. William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. Breast Cancer Res Treat 44(1):23-38.

Klotz DM, Hewitt SC, Korach KS, Diaugustine RP. 2000. Activation of a uterine insulin-like growth factor I signaling pathway by clinical and environmental estrogens: requirement of estrogen receptor-alpha. Endocrinology 141(9):3430-3439.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139(10):4252-4263.

Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, et al. 2000. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74(5):311-317.

Li Y, Burns KA, Arao Y, Luh CJ, Korach KS. 2012. Differential Estrogenic Actions of Endocrine-Disrupting Chemicals Bisphenol A, Bisphenol AF and Zearalenone through Estrogen Receptor alpha and beta in Vitro. Environ Health Perspect 120(7):1029-1035.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90(23):11162-11166.

Markaverich BM, Webb B, Densmore CL, Gregory RR. 1995. Effects of coumestrol on estrogen receptor function and uterine growth in ovariectomized rats. Environ Health Perspect 103(6):574-581.

Matsushima A, Kakuta Y, Teramoto T, Koshiba T, Liu X, Okada H, et al. 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. J Biochem 142(4):517-524.

Matsushima A, Liu X, Okada H, Shimohigashi M, Shimohigashi Y. 2010. Bisphenol AF is a full agonist for the estrogen receptor ERalpha but a highly specific antagonist for ERbeta. Environ Health Perspect 118(9):1267-1272.

McDonnell DP, Norris JD. 2002. Connections and regulation of the human estrogen receptor. Science 296(5573):1642-1644.

Mueller SO, Hall JM, Swope DL, Pedersen LC, Korach KS. 2003. Molecular determinants of the stereoselectivity of agonist activity of estrogen receptors (ER) alpha and beta. The Journal of biological chemistry 278(14):12255-12262.

Murray TJ, Lea RG, Abramovich DR, Haites NE, Fowler PA. 2001. Endocrine disrupting chemicals: effects on human male reproductive health. Early Pregnancy 5(2):80-112.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. 2001. Mechanisms of estrogen action. Physiol Rev 81(4):1535-1565.

O'Lone R, Frith MC, Karlsson EK, Hansen U. 2004. Genomic targets of nuclear estrogen receptors. Mol Endocrinol 18(8):1859-1875.

Perez P, Pulgar R, Olea-Serrano F, Villalobos M, Rivas A, Metzler M, et al. 1998. The estrogenicity of bisphenol A-related diphenylalkanes with various substituents at the central carbon and the hydroxy groups. Environ Health Perspect 106(3):167-174.

Pettersson K, Gustafsson JA. 2001. Role of estrogen receptor beta in estrogen action. Annu Rev Physiol 63:165-192.

Reid G, Metivier R, Lin CY, Denger S, Ibberson D, Ivacevic T, et al. 2005. Multiple mechanisms induce transcriptional silencing of a subset of genes, including oestrogen receptor alpha, in response to deacetylase inhibition by valproic acid and trichostatin A. Oncogene 24(31):4894-4907.

Sharma SC, Richards JS. 2000. Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. J Biol Chem 275(43):33718-33728.

van Lipzig MM, ter Laak AM, Jongejan A, Vermeulen NP, Wamelink M, Geerke D, et al. 2004. Prediction of ligand binding affinity and orientation of xenoestrogens to the estrogen receptor by molecular dynamics simulations and the linear interaction energy method. J Med Chem 47(4):1018-1030.

Webb P, Lopez GN, Uht RM, Kushner PJ. 1995. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9(4):443-456.

Welshons WV, Nagel SC, vom Saal FS. 2006. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147(6 Suppl):S56-69.

Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. 2007. In vitro molecular mechanisms of bisphenol A action. Reprod Toxicol 24(2):178-198.

Ying GG, Williams B, Kookana R. 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates--a review. Environ Int 28(3):215-226.

Table 1: Endocrine disrupting chemicals (EDCs) used in this study

EDCs	Chemical class	Product class	MW
17β-estradiol (E2)	Steroid, phenolic; Estrene	**Hormone**	272.38
ICI 182,780 (ICI)	Steroid, phenolic	Pharmaceutical	606.77
Group 1			
Bisphenol A (BPA)	Diphenylalkane; Bisphenol; Phenol	Chemical intermediate	228.29
Bisphenol AF (BPAF)	Diphenylalkane; Bisphenol; Phenol	Chemical intermediate	336.23
2-2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)	Diphenylalkane; Bisphenol; Phenol	Chemical intermediate	317.59
4-n-Nonylphenol (4-n-NP)	Alkylphenol; Phenol	Chemical intermediate	220.35
Group 2			
Daidzein (Dai)	Flavanoid; Isoflavone; Phenol	Natural product	254.23
Genistein (Gen)	Flavanoid; Isoflavone; Phenol	Natural product	270.24
Kaempferol (Kaem)	Flavanoid; Isoflavone; Phenol	Natural product	286.23
Apigenin (Api)	Flavanoid; Flavones; Phenol	Natural product	270.24
Coumestrol (Coum)	Flavanoid; Isoflavone; Phenol	Natural product	282.22
Group 3			
Endosulfan (Endo)	Organochlorine	Pesticide	406.93
Kepone (Kep)	Organochlorine	Pesticide	490.64
1-Bromopropane (1-BP)	Organochlorine	Chemical intermediate	122.99

Figure Legends

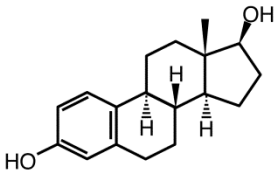
Figure 1. The chemical structures of EDCs tested in this study.

Figure 2. EDCs act as agonists on ERα and ERβ to activate the classical mechanism (ERE) in HepG2 and HeLa cells. (A) Activation of ERα. Cells were transfected with ERE-luc (3xERE or pS2 ERE), pRL-TK and pcDNA/WT-ERα plasmids overnight. After changing to fresh starve medium, cells were treated with the vehicle (control), 10 nM E2, 100 nM ICI, or EDCs for 18 hours. ERα ERE-mediated activation was detected by luciferase reporter assays as described in Material and Methods. Data shown is representative of triplicates as fold increase calculated relative to the vehicle (control) ± SEM, ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to vehicle (control). (B) Activation of ERβ. Cells were transfected with ERE-luc (3xERE or pS2 ERE), pRL-TK and pcDNA/WT-ERβ plasmids overnight. After changing to fresh starve medium, cells were treated with the vehicle (control), 10 nM E2, 100 nM ICI or EDCs for 18 hours. ERβ ERE-mediated activation was detected by luciferase reporter assays as described in Material and Methods. Data shown is representative of triplicates as fold increase calculated relative to the vehicle (control) ± SEM, ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to vehicle (control).

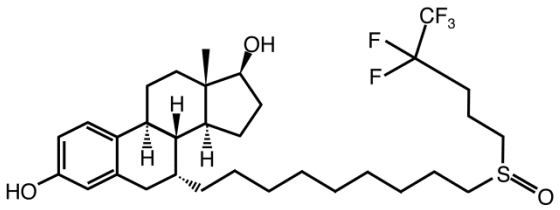
Figure 3. EDCs act as agonists on ERα and ERβ to activate the tethered mechanism (AP-1). (A) Effects on ERα 7xAP-1 Luc reporter activity in HepG2 and HeLa cells. Cells were transfected with 7xAP-1 Luc, pRL-TK, pcDNA/WT-ERα or -ERβ and pRSV/c-Jun plasmids overnight. After changing to fresh starve medium, cells were treated with the vehicle (control), 10 nM E2, 100 nM ICI or EDCs for 18 hours. ER AP-1-mediated activation was detected by luciferase reporter assays as described in Material and Methods. Data shown is representative of triplicates

as fold increase calculated relative to the vehicle (control) ± SEM, ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to vehicle (control). (B) Effects on ERα and ERβ -73Col AP-1 Luc reporter activity in HeLa cells. Cells were transfected with -73Col AP-1 Luc, pRL-TK, and pcDNA/WT-ERα or -ERβ plasmids overnight. After changing to fresh starve medium, cells were treated with the vehicle (control), 10 nM E2, 100 nM ICI, or EDCs for 18 hours. ER -73Col AP-1-mediated activation were detected by luciferase reporter assays as described in Material and Methods. Data shown is representative of triplicates as fold increase calculated relative to the vehicle (control) ± SEM, ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to vehicle (control).

Figure 4. The effects of EDCs on the ER target gene expression of *PR*, *pS2*, *GREB1, SPUVE*, *WISP2*, and *SDF-1* in Ishikawa/vector and Ishikawa/ERα cells. Total RNA was extracted from Ishikawa/vec or Ishikawa/ERα cells after the vehicle (control), 10 nM E2, 100 nM ICI, or EDCs treatments for 18 hours. mRNA levels of *PR*, *pS2*, *GREB1*, *SPUVE*, *WISP2*, and *SDF-1* were quantified by real time-PCR. Data shown is representative of triplicates as fold increase calculated relative to the vehicle (control) ± SEM, ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared with the vehicle (control) of Ishikawa/vec cells.

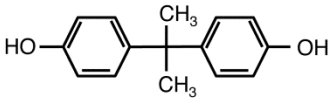


17β-estradiol (E2)

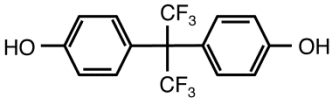


ICI 182,780 (ICI)

Group 1



Bisphenol A (BPA)



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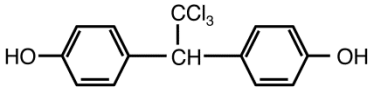
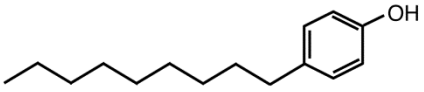
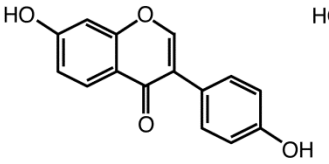


2-2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)

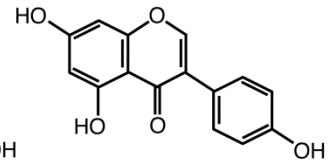


4-n-nonylphenol (4n-NP)

Group 2



Daidzein (Dai)



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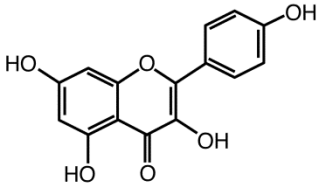
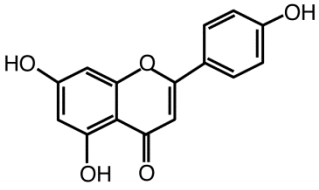
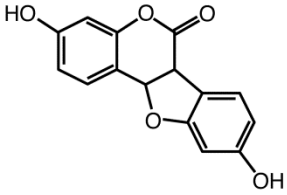


Kaempferol (Kaem)

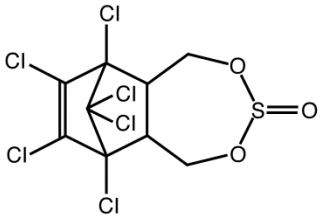


Apigenin (Api)

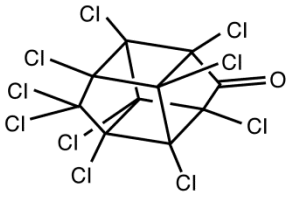


Coumestrol (Coum)

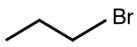
Group 3



Endosulfan (Endo)



Kepone (Kep)



1-Bromopropane (1-BP)

Figure 1

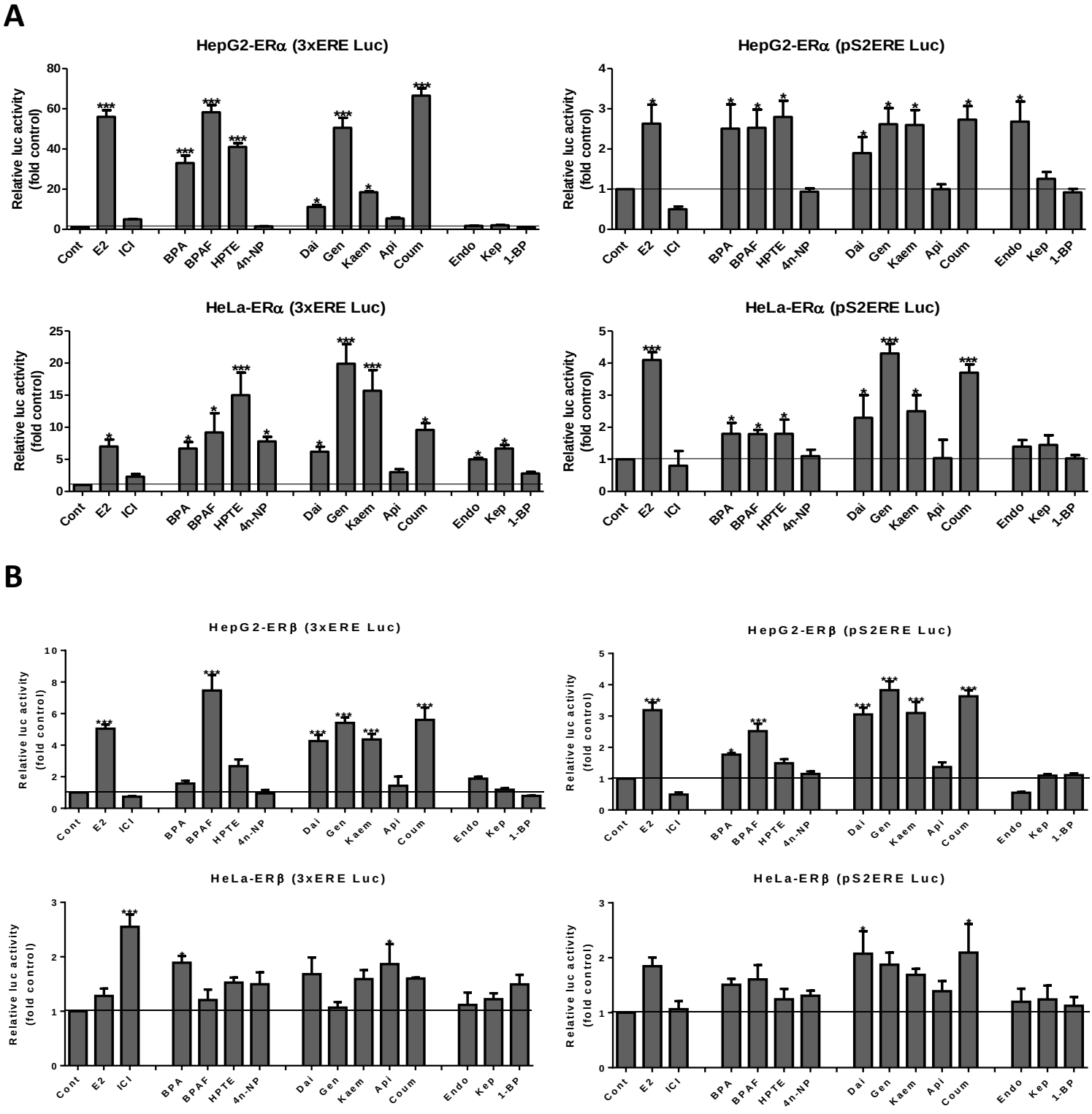

A
HepG2-ERα (3xERE Luc)
HepG2-ERα (pS2ERE Luc)
HeLa-ERα (3xERE Luc)
HeLa-ERα (pS2ERE Luc)
B
HepG2-ERβ (3xERE Luc)
HepG2-ERβ (pS2ERE Luc)
HeLa-ERβ (3xERE Luc)
HeLa-ERβ (pS2ERE Luc)
Relative luc activity (fold control)
Cont
E2
ICI
BPA
BPAF
HPTE
4n-NP
Dai
Gen
Kaem
Api
Coum
Endo
Kep
1-BP

Figure 2

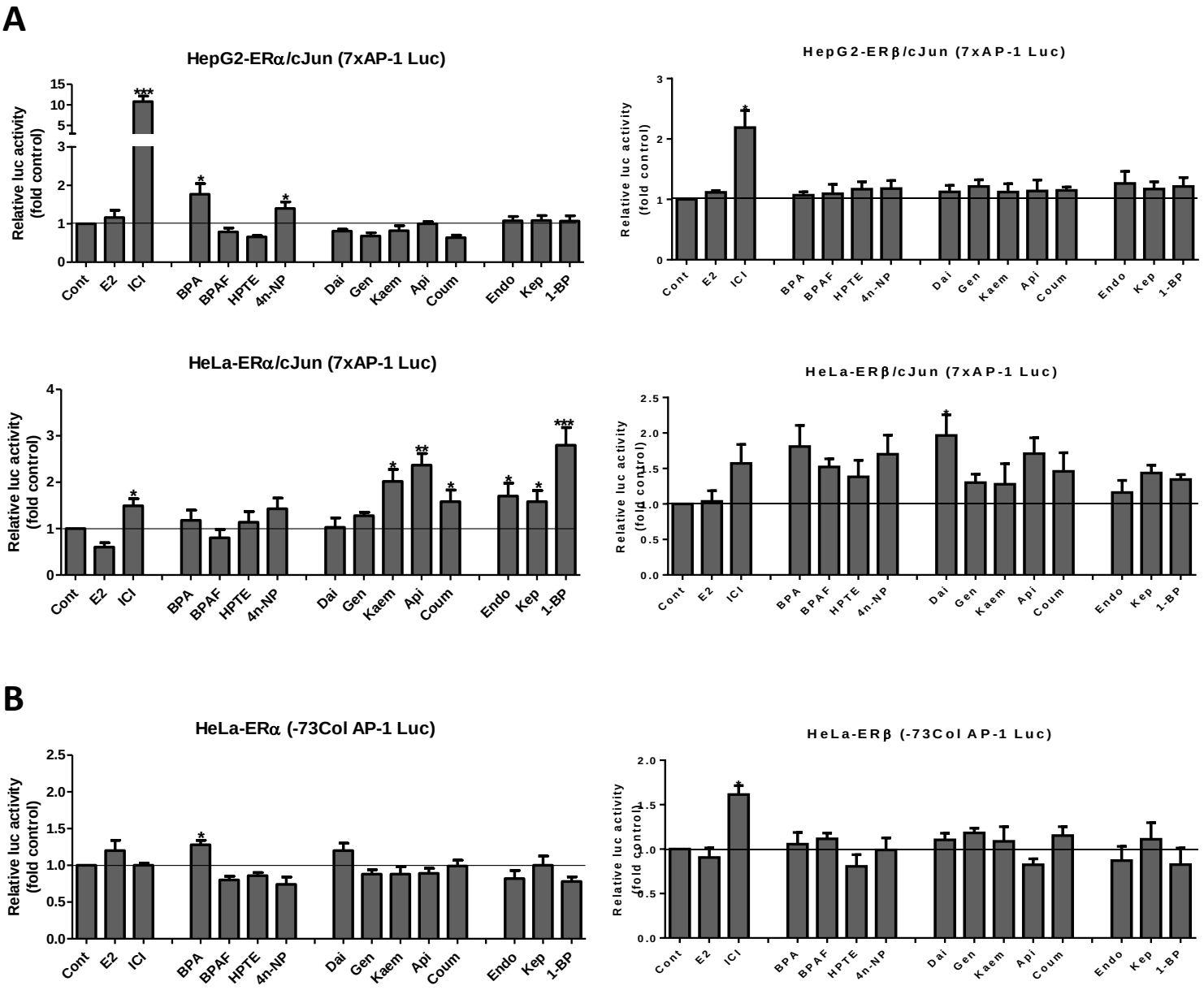

A
HepG2-ERα/cJun (7xAP-1 Luc)
HepG2-ERβ/cJun (7xAP-1 Luc)
HeLa-ERα/cJun (7xAP-1 Luc)
HeLa-ERβ/cJun (7xAP-1 Luc)
B
HeLa-ERα (-73Col AP-1 Luc)
HeLa-ERβ (-73Col AP-1 Luc)
Relative luc activity (fold control)
Cont
E2
ICI
BPA
BPAF
HPTE
4n-NP
Dai
Gen
Kaem
Api
Coum
Endo
Kep
1-BP

Figure 3

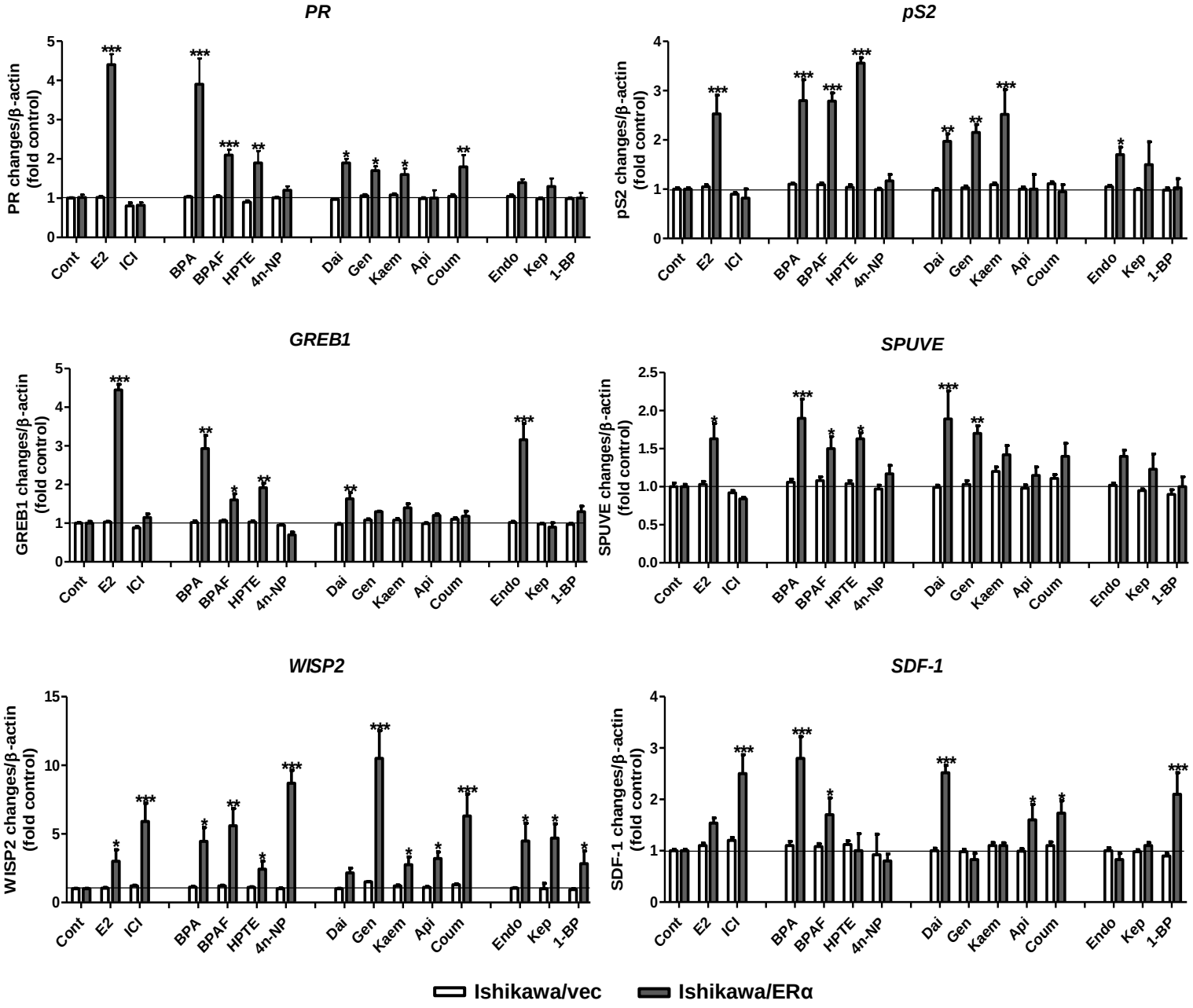

PR
PR changes/β-actin (fold control)
pS2
pS2 changes/β-actin (fold control)
GREB1
GREB1 changes/β-actin (fold control)
SPUVE
SPUVE changes/β-actin (fold control)
WISP2
WISP2 changes/β-actin (fold control)
SDF-1
SDF-1 changes/β-actin (fold control)
Cont
E2
ICI
BPA
BPAF
HPTE
4n-NP
Dai
Gen
Kaem
Api
Coum
Endo
Kep
1-BP
Ishikawa/vec
Ishikawa/ERα

Figure 4